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GRANT NUMBER DAMD17-96-1-6177

TITLE: Regulation of Estrogen Receptor mRNA Stability in Human Breast Cancer Cells

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REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
Regulation of Estrogen Receptor mRNA Stability in Human Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-96-1-6177

6. AUTHOR(S)
Vincent DiPippo, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Illinois
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The growth and metastases of approximately 40% of human breast cancers is dependent on 17β-estradiol-estrogen receptor (E_2-ER) complex. ER levels are controlled, in part, through an autoregulatory loop in which E_2-ER complex (and mitogens) down-regulate ER mRNA levels - a process I have studied using Northern blot hybridization of RNAs extracted from MCF-7 human breast cancer cells. To resolve the ongoing controversy over the extent to which ER mRNA detabilization contributes to down-regulation, I had to develop new techniques for measuring mRNA degradation. I have created tetracycline regulated expression plasmids which transcribe either the full length 6.4 kb human ER mRNA or truncated 'mini-ER' mRNAs, identified improved methods for delivering the plasmids encoding these mRNAs to cells in transient transfections, and developed a novel quantitative PCR procedure, which for the first time in any system, allows quantitative PCR of RNA from cells transiently transfected with DNA plasmids encoding the RNA.

Using this system, I will determine the specific sequences in ER mRNA important in estrogen and TPA destabilization and identify proteins binding to these sequences. This will provide important new information on mechanisms controlling ER mRNA levels in breast cancer cells.

14. SUBJECT TERMS Breast Cancer
Estrogen Receptor, mRNA degradation, MCF-7 Breast Cancer Cells, Estrogen, Phorbol Ester, mRNA Binding Protein

15. NUMBER OF PAGES
34

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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Vincent A. DePaso 9-29-97

PI - Signature Date

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INTRODUCTION

A. Estrogen-Dependent Breast Cancer.

The estrogen receptor (ER) mediates the actions of estrogens in target tissues. Estrogens acting through the ER, strongly influence the growth, proliferation and metastatic potential of breast cancers (1,2). Approximately half of all breast cancers contain high levels of ER. The growth of approximately 2/3 of these tumors is strongly dependent on the presence of the estrogen-ER complex (3-5). Although antiestrogens such as tamoxifen are widely used to slow the growth of these tumors, the progression of tumors to hormone-independent growth limits the effectiveness of tamoxifen therapy (6-8).

B. Control of mRNA Degradation.

An important action of estrogens, androgens and other steroid hormones is the post-transcriptional regulation of messenger ribonucleic acid (mRNA) stability. Although steroid hormones regulate the stability of numerous vertebrate mRNAs (9-11), the mechanisms responsible for this regulation have remained largely obscure. Two simple models have been proposed. A nuclear or cell surface receptor could regulate the stability of a specific RNA either by controlling the interaction of a binding protein with the mRNA, or by regulating the activity of nucleases responsible for mRNA degradation. Consistent with the binding protein model is the observation that estrogen specifically induces the 155 kD protein, vigilin (12), which binds specifically to a segment of the vitellogenin mRNA 3' untranslated region (3' UTR) essential for estrogen-stabilization (13,14). Only in a few systems have specific sequences or regions of mRNA been identified as the sites affected by hormonal regulation of mRNA stability (10,15). Phorbol ester regulation of a binding factor to AUUUA motifs in the unstable granulocyte macrophage-colony stimulating factor (GM-CSF) mRNA appears to be mediated through a protein kinase C-dependent pathway (16). Protein kinase C is also implicated in the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induced decrease in ER mRNA in MCF-7 cells (17), but the exact mRNA sites affected remain to be identified.

While sequences which regulate mRNA stability have been detected at a variety of locations within mRNAs, most studies have focused on sequences

within the 3' UTR (18-20). In at least two well characterized systems, cis-acting elements in the 3' UTR have been shown to interact with binding proteins to regulate mRNA stability (10,15). Similarly, sequences in the 3' UTR have been implicated in both the estrogen regulation of vitellogenin mRNA stability and the regulation of mRNA stability by thyroid hormone (10,15).

One of the key factors implicated in the control of ER levels in breast cancer cells is an autoregulatory loop in which the estrogen-ER complex (E_2-ER) down regulate ER mRNA levels (21-25). At least part of this down-regulation is achieved by E_2-ER dependent destabilization of ER mRNA. mRNA stability has been reported to be an important factor in controlling ER levels in MCF-7 breast cancer cells (17,26,27). However, little is known of the mechanisms by which regulation occurs, and the extent to which ER mRNA destabilization contributes to down-regulation of ER mRNA levels remains controversial (17,21-32). Estrogens, antiestrogens and tumor promoters can all play a critical role in determining the stability of ER mRNA in breast cancer cells (17,26,27). Consequently, they may have a significant effect on the level of ER protein that is ultimately expressed in breast cancer cells. Since the level of ER present in breast cancer cells will be an important factor in the determination of the course of patient therapy, the goals of this project are to determine the contribution of ER mRNA destabilization on down-regulation of ER mRNA levels, and to identify the protein(s) and ER mRNA binding sites which are responsible for the regulation of ER mRNA stability.

I will determine the extent of destabilization of ER mRNA mediated by estrogen and TPA, identify the region of the 3' UTR important in ER mRNA destabilization and determine if sequence specific RNA binding proteins bind to the essential region of the ER mRNA.

Determining mRNA degradation rates using inhibitors of RNA synthesis, such as actinomycin D (AMD), is relatively straightforward. However, these methods are prone to artifacts (33,34). While several laboratories have used these simple, but imperfect, methods to demonstrate estrogen or TPA dependent destabilization of ER mRNA, the extent of the destabilization had been controversial (17,28-32). Previous work done by others and reproduced in our lab within the past year has not explained the mechanism for the down-

regulation of ER mRNA. Perhaps because of the large size (6.4 kb) of ER mRNA, there is presently no information on the mRNA sequences important in ER mRNA destabilization. Since the available technology for studying ER mRNA degradation produced data which was not definitive, a major undertaking this year was to develop a new methodology for studying ER mRNA degradation. This technology should be widely applicable to studies of both mRNA synthesis and degradation. The several components of this system which are detailed below include: (i) An improved system for introducing DNA into MCF-7 human breast cancer cells. (ii) A full length (6.4 kb) ER cDNA clone transcribed into mRNA under the control of the regulated tetracycline expression system. This eliminates the need for global RNA synthesis inhibitors, such as AMD, because synthesis of the test RNA can be specifically turned off. (iii) A quantitative reverse transcriptase polymerase chain reaction (RT-PCR) procedure for highly accurate quantitation of mRNA transcribed from the tetracycline (TET)-regulated expression plasmid. This was a formidable undertaking since, to our knowledge, there are no reports of successful quantitative RT-PCR of RNA transcribed from transfected plasmid DNA. The transfected DNA is present in excess and is amplified as a contaminant in the RT-PCR reaction. Unusual amplification and purification techniques were employed to successfully surmount this problem (see below).

The scope of this research includes: (i) Using Northern blot hybridization to confirm that estrogen and TPA down-regulate ER mRNA levels. (ii) Developing the new system for investigating ER mRNA degradation described above. (iii) Determining the degradation rate of ER mRNA in response to estrogens and TPA. (iv) Determining the region of ER mRNA which is required for estrogen destabilization. I will construct and test mRNA deletions and replacements to determine whether sequences in the 5' UTR, the coding sequence, and as is most probable, the 3' UTR of human ER mRNA can be removed or replaced without abolishing estrogen-stimulated degradation. The constructions will be prepared in the regulated tetracycline expression system (35), and assayed using the system described above. (v) I will determine whether the segments of the mRNA important for stability regulation exhibit sequence specific binding to either the human homologue of the estrogen-regulated,155 kD mRNA binding protein, vigilin, or other MCF-7 cell proteins.

RNA gel mobility shift assays (13) will be used to determine whether the mRNA sequences important in estrogen-destabilization exhibit a sequence specific interaction with vigilin or with other cellular proteins. I will also determine if estrogen or phorbol ester, which alter ER mRNA stability, influence binding of the protein to the mRNA. (vi) I will determine whether estrogen and phorbol esters destabilize ER mRNA through a common mechanism. I will determine if the effects of estrogen and TPA are either additive or synergistic when given in combination.

BODY

Experimental Methods, Assumptions, and Procedures

Northern Blot Assays

Total RNA was isolated from MCF-7 cells with Trizol (Gibco BRL) reagent as per manufacturer's instructions (36). 5 μg of each RNA sample were resolved on a 1% formaldehyde denaturing agarose gel. After 4 to 6 hours, the gel was stained in 33 mg/ml acrydine orange for 10 minutes while shaking. After destaining, the gel was photographed and then blotted to a nylon membrane (Pall Corporation) overnight in sterile 20 x SSC. After baking for 2 hours at 80°C and U.V. exposure, the blot was prehybridized at 42°C for 2 to 4 hours and then cohybridized with radiolabeled cDNA probes as previously described (37). ER and 36B4 cDNA were radiolabeled with [^{32}P]dCTP by random priming using standard methods. 36B4 mRNA served as an internal control. The level of 36B4 mRNA is constant, and is not modified by either estrogen or TPA (38). Intensities of labeled bands were determined using a PhosphorImager (Molecular Dynamics).

Whole Cell ER Assay

ER assays were carried out as previously described (37) with minor modifications. Briefly, 1 x 10^6 MCF-7 cells were seeded and on the following day were incubated in the presence or absence of 10 nM TPA for 24 hours. The cells were harvested and transferred to polypropylene tubes containing 1 ml of 1x Minimum Essential Medium (MEM) and 10 nM [^{3}H]E_2 (Amersham Corp.) in ethanol or 10 nM [^{3}H]E_2 plus 1 mM unlabeled E_2. After a 1 hour incubation at 37° C, the cells were pelleted and washed three times with cold 1x Tris buffered saline (TBS), 33 mM Tris, pH 7.4, 150 mM NaCl, containing 1% Tween 80. The pellets were resuspended in 1 x TBS and counted in 10 ml if Triton/xylene fluor.

Cell Culture, Transient Transfection Assays and CAT Assays

MCF-7 cells are maintained as previously described (39). MDA-MB-231 (231N) cells are maintained in 1x Leibovitz's L-15 media with 10 mM HEPES, pH 7.4, 5% calf serum, 100 units penicillin/ml, 100 μg streptomycin/ml, 25 μg gentamycin/ml, 6 ng bovine insulin/ml, 3.75 ng hydrocortisone/ml, 16 μg glutathione/ml. For experiments including estrogen, MCF-7 or 231N cells were first placed in MEM (Sigma) or L-15 media (Gibco BRL), respectively, maintained in medium containing 5% charcoal-dextran stripped calf serum for 5 days and then placed in MEM or L-15 media, respectively, minus phenol red with 5% charcoal-dextran stripped calf serum for 3 to 5 days prior to treatment. Each transfection method (calcium phosphate precipitation (22,39)), HMG protein gene delivery agent (40), Clonfectin transfection reagent (Clontech), SuperFect transfection reagent (Qiagen), Transfectam transfection reagent (Promega), and electroporation (41) was performed as per authors' or manufacturers' instructions. CAT activity was determined by quantitative mixed-phase assay as previously described (42).

For the quantitative (QT)-RTPCR experiments, in order to eliminate effects due to variations in transfection efficiency within an experiment, I will transfect the cells; the cells will then be trypsinized, harvested, and replated onto two plates, one receiving estrogen or TPA and the other minus estrogen or TPA. Reproducibility is enhanced since both plates are derived from the same transfection.

Tetracycline (TET)-expression system

The TET expression system developed by Gossen and Bujard (35), which produces tight control of gene expression by tetracycline regulated promoters, has been successfully implemented in our lab. In this system, the addition of a tetracycline antibiotic to the medium of MCF-7 or 231N cells activates binding of a TET repressor-VP16 fusion protein to the operator and inactivates transcription. After shutting off transcription, MCF-7 or 231N cells will receive either vehicle or estrogen and/or TPA. The cells are then harvested, RNA is isolated and QT-RTPCR experiments are used to determine differences in ER mRNA levels.

Plasmid Constructions

All oligonucleotides used were synthesized at the University of Illinois Biotechnology Center and purified with oligonucleotide purification cartridges (Applied BioSystems). To create the tetracycline regulated full-length 6.4 kb human ER (TET-flhER) cDNA construction, an RTPCR reaction was performed. The RT reaction was performed with total RNA from MCF-7 cells and a 3'-primer (5'AGGGAATTCCTTACAAAAAACAAACTCGTTGTCTTTAGTTTAATTCTTTATT TGAACATCAAATAGG3') which was specific for the most downstream 3' portion of the ER mRNA including a small portion of the 3' flanking region which contains the polyadenylation signal found in the cDNA of the ER. Superscript reverse transcriptase (RT) (Gibco BRL) was used for all RT reactions as described in the manufacturer's protocol. Because of the long size of the PCR product, PCR reactions were attempted using *Taq* (Gibco BRL), *Pfu* (Stratagene) or Elongase (Gibco BRL) DNA polymerases. The PCR portion of this reaction utilized the same 3' primer that was used in the RT reaction and a 5' primer (5'TAGGAATTCCAGGAGCTGGCGGAGGGCGTTCG3') which recognized the most upstream portion of the ER cDNA coding for the ER mRNA 5' UTR. This PCR product was digested with EcoRI and ligated into an EcoRI digested modified vector containing a tetracycline-controlled transactivator (tTA)-dependent promoter (pUHD10-3) (kindly provided by Dr. H. Bujard) using a Takara ligation kit. All subsequent TET-mini-ER constructions are the result of various restriction digest modifications of the initial TET-flhER plasmid.

A construct was created as an internal standard for QT-RTPCR experiments. The TET-flhER plasmid was digested with BglII and EcoRI and run on a 1% low melt agarose gel. A fragment containing the cDNA coding for the last 889 nucleotides of the ER mRNA 3'UTR was gel purified and cloned into a BamHI and EcoRI digested pGEM3 expression vector. This clone was then digested with XhoI and ligated with a XhoI digested 67 nucleotide PCR product. The 67 nucleotide PCR product was generated using a pSP72 (Promega) vector, a 5' primer recognizing the SP6 promoter, and a 3' primer targeted to the multiple cloning site (MCS) of the pSP72 vector.

I am currently constructing a number of TET-mini-ER plasmids to determine which of these when transcribed are sufficient to confer estrogen destabilization in MCF-7 and 231N cells. While some of these constructions are not trivial, a similar approach was used successfully on vitellogenin mRNA in this

laboratory (10). Since most known mRNA destabilizing sequences are found in 3'-UTR, the first few mini-ER constructions will test this area.

RTPCR Experiments

RTPCR experiments were performed using total RNA from MCF-7 and 231N cells, a 3' primer (5' CTCAGACTGTGGCAGGGAAACCCTCTGCCTC3') which recognizes the ER cDNA which codes for the C-terminus of the protein and a 5' primer (5'AATGACTATGCTTCAGGC3') which binds to the ER cDNA which codes for the DNA binding domain. PCR reactions were performed with *Taq* polymerase (Gibco BRL) and generated an approximately 1.2 kb product.

QT-RTPCR Experiments

Our lab has recently set up and implemented a modification of existing QT-RTPCR methodology (43-45). Due to the technical challenges of measuring mRNA levels generated from plasmids that were transiently transfected into breast cancer cells, a DNase protocol has been included in our procedure (46). A second effective method we have employed to prevent plasmid cDNA from being PCR amplified, involves using a 'tag' 3' primer in the PCR reaction which will only recognize ssDNA which was reversed transcribed. This modification has been developed (47) and used successfully in our laboratory. The mRNA used as an internal standard is produced using an *in vitro* transcription system using an SP6 regulated promoter and radiolabeled for quantitative purposes.

Results and Discussion

The essential feature of these studies is to prepare recombinant DNA constructions which are tetracycline-regulated and compare the degradation of the ER mRNA transcribed from the sequence in transiently transfected breast cancer cells in the presence or absence of 17β-estradiol or phorbol ester.

Preliminary Studies (Northern Blot Assays and Whole Cell ER Assays)

The use of an experimental system in which production of the mRNA can be shut off, greatly simplifies measurement of mRNA degradation. Although AMD is widely used in studies of mRNA stability, this laboratory and others (33,34) find that it can give artifactual measurements of mRNA half lives. Nevertheless, my initial studies involving northern blot experiments were performed in order to determine: a) whether the MCF-7 breast cancer cells, that I will use in future experiments, would behave in a manner consistent with what others have shown (17,21-31) with respect to their responsiveness to estrogen or TPA and b) whether, in the process of replicating previous AMD experiments performed by others, the northern blot assay would be sensitive enough to detect relatively low mRNA levels generated from the transiently transfected tetracycline regulated expression system.

Initial experiments in the absence of AMD with both estrogen and TPA confirmed previously published reports that both of these agents dramatically lower ER mRNA levels in MCF-7 cells. Northern blots confirmed that after 24 hours, greater than 80% decreases in ER mRNA were seen at concentrations of 10 nM for estrogen and TPA when compared to untreated MCF-7 cells (Fig 1) . This was our first piece of evidence that the MCF-7 cells which I will use for future experiments, exhibit down-regulation of ER mRNA by TPA and estrogens. In collaboration with Maria Acena, an MD/PhD student in our lab, we have performed whole cell ER assays which show that the decrease in ER mRNA levels is reflected in a decrease in ER protein (120,440$\pm$7080 ER sites/cell (-TPA, n=3) vs. 44,202$\pm$2129 ER sites/cell (+TPA, n=3)). In experiments where a TET- or CMV-expression vector containing the cDNA for the 2.0 kb ER protein coding region was transfected into MCF-7 cells via electroporation, the transcribed 2.0 kb ER mRNA was undetectable using the Northern blotting procedure (data not shown). This was not surprising as the efficiency of transfection of MCF-7 cells is low, and, using simple Northern blot hybridization, it has not generally been possible to directly detect mRNA from transfected regulated promoters.

I next carried out experiments using the transcriptional inhibitor AMD in the presence and absence of TPA. This was done in order to look at ER mRNA stability using widely employed standard methods. Two time course experiments were completed. The first was based on the protocol of Ree et al (30), where

MCF-7 cells where cultured with: TPA (100 nM) alone, AMD (4 μM), TPA+AMD, or vehicle control. As is shown in Figure 2, all three non-vehicle treated groups rapidly lowered ER mRNA levels within 3 hours. However, no differences were seen when comparing the degradation rates of these groups. In the second time course experiment (Fig. 3), the protocol was based on that of Saceda et al (17). 24 hours after treatment with either TPA or vehicle, transcription was stopped by the addition of 4 μM AMD. The results of this experiment were similar to those of their group and give supportive evidence to the finding that TPA can decrease ER mRNA levels by destabilizing the message. A significant difference between the two time course experiments is the time at which the AMD is administered. In the first experiment both TPA and AMD are administered at the same time while in the second, the AMD is given after the cells were exposed to TPA for 24 hours. This most likely accounts for the differences between the two results. These results serve to emphasize our need for developing a system which can address mRNA stability while regulating only the specific mRNA of interest.

Transient Transfection Assays

While our lab has successfully transfected (39) both the ER positive MCF-7 cell line and the ER negative 231 N breast cancer cell line using the calcium phosphate precipitation method, the efficiency of transfection of the MCF-7 cells is quite low. My experiments involve analysis of mRNAs transcribed from DNA constructs inserted into MCF-7 cells by transient transfection. To identify an efficient method for transfecting MCF-7 cells, I carried out a detailed test of several different transfection methods and reagents. In these studies I analyzed the activity of CMV-CAT reporter plasmid transfected in to the cells. Based on the results of these experiments (Figs. 4-7), I am currently using electroporation as the optimal method for transfecting MCF-7 cells. Prior to working on this project, I consistently and effectively used the calcium phosphate precipitation method of transient transfection in a number of cell lines (48) and will therefore continue to use this method with the 231N cells.

RTPCR and Cloning Strategies

A significant difference between MCF-7 and 231N breast cancer cells is their respective presence and absence of endogenous ER. With respect to ER

mRNA, while it is readily detectable in MCF-7 cells through conventional Northern blotting, it can not be detected in a similar manner in 231N cells. Taking advantage of the more sensitive mRNA detection method of RTPCR, an initial RTPCR experiment was performed which determined that 231N cells contained no detectable levels of ER mRNA (Fig. 8).

The absence of significant mechanistic data on ER mRNA degradation is due in large part to the difficulty of obtaining and working the full-length 6.4 kb ER cDNA clone. Since deciding to develop a new strategy for measuring mRNA stability using QT-RTPCR, it was essential to obtain such a clone for insertion into the regulated expression plasmid. While some clones currently exist which contain the protein coding region of the ER or fragments of the approximately 4 kb 3' UTR of the ER, I was unable to locate any clone in the literature which contains the entire 5' UTR, the protein coding region and the entire 3'UTR of the ER. I, therefore, decided to use long-RTPCR to create a full length human ER-TET (TET-flhER) vector (Fig 9). This was done so that the TET regulated ER construction would produce a 3' UTR which is identical to that of endogenous ER mRNA. However, the PCR reaction was unsuccessful with both *Taq* and *Pfu* polymerase. After unsuccessfully attempting to optimize the conditions for the PCR reactions using these polymerases, I eventually was able to make the full length PCR product using the Elongase enzyme. The identity of the 6.4 kb product as ER cDNA was verified through a number of restriction digests. A segment of this construction was then used to create an internal standard necessary for QT-RTPCR. The internal standard has a 67 nucleotide insert within the ER mRNA 3' UTR which serves to create a PCR product which is produced from the same primers as the test sequence, but is of a distinct molecular weight (Fig. 9).

Although the TET regulated 6.4 kb full length ER cDNA is identical to the endogenous ER mRNA after transcription, distinguishing the TET derived transcript from the endogenous ER mRNA is only an issue in the MCF-7 cells. Since I have demonstrated that 231N cells contain no detectable endogenous ER mRNA, they can therefore be transfected with the TET-flhER plasmid unmodified. For MCF-7 cells however, the TET-flhER was slightly modified so that it is unique from the endogenous ER mRNA and can be recognized exclusively in subsequent QT-RTPCR experiments using a primer which binds to the unique site. While this approach has precluded me from going straight to a

"mini-ER" as I originally proposed, using the more sensitive technology of QT-RTPCR will allow me to proceed more rapidly than with less sensitive assays and will greatly facilitate rapid work on the projects objectives. Moreover, by first testing the full length ER mRNA, I believe our improved experimental design has become more systematic and therefore more informative as well.

After testing the 6.4 kb full length ER mRNA in both 231N and MCF-7 cells, I will proceed rapidly to a mini-ER mRNA which does not contain most of the 4 kb 3' UTR found in ER mRNA (49,50). This approach is feasible. Although mRNA destabilization sequences can occur anywhere in an mRNA, most destabilization sequences occur in the 3'UTR because most known mRNA destabilizing sequences lie within a few hundred nucleotides of the 3'-end of their mRNAs. Since the QT-RTPCR system is up and running, as soon as the constructions are made they can be transfected and subsequently tested. Finally, a highly accurate PhosphorImager (51) is used to quantitate the radioactive bands on the gels. With several replicates of each experiment, highly accurate data can be obtained-even for effects which are not quantitatively large. My subsequent studies will follow the same general scheme used in this laboratory to determine that more than 90% of the vitellogenin mRNA sequence was not required for estrogen stabilization (10). If successful, this will rapidly eliminate from consideration most of the sequence of the 6.4 kb ER mRNA.

QT-RTPCR

As well as the Northern blot assay, ER mRNA levels could be also be monitored in conventional dot hybridizations (52) or by using the S_1 nuclease protection assay with internal standards (10). However, the sensitivity of these methods became more of a concern as I conducted the Northern blot experiments and increased the transfection efficiency in MCF-7 cells. The decision to switch to QT-RTPCR as a method for detecting differences in mRNA levels, has made it unnecessary to maximize the sensitivity of the previously mentioned RNA detection methods. These reactions will produce quick and immediate results (in as little as one day) and definitively determine the effects of estrogen and/or TPA on ER mRNA at the post-transcriptional level.

Recommendations in Relation to the Statement of Work

The statement of work was prepared for a two year time frame with only the first of four tasks to be completed in the first 12 months. The decision to set up and develop a novel QT-RTPCR, for measuring a more sensitive technology for detecting mRNA from transfected cells, has resulted in only a partial completion of the stated objectives of the first task (Months 1-12: I Will Determine the Region of ER mRNA which Is Required for Estrogen Destabilization.). Technical hurdles such as maximizing transfection efficiency in MCF-7 cells and the establishment in our lab of a new mRNA detection protocol were unfortunately time consuming but nevertheless, necessary. However, the improved strategy will now result in a quicker time frame for completing the first task as mini-ER mRNA can now be evaluated in less than one day after RNA extraction from breast cancer cells with QT-RTPCR.

The second task (Months 12-24: I will determine whether the segments of ER mRNA important for stability regulation contain binding sites for the MCF-7 homologue to the 155 kD estrogen-regulated *Xenopus* mRNA binding protein (vigilin), or whether other breast cancer cell proteins exhibit sequence specific binding to this sequence.) is still applicable and will be accomplished immediately after task 1. Others in our lab are currently working on expressing significant amounts of purified human vigilin protein which I can test directly in ER mRNA binding studies.

Fortunately, due to the work of Robin Dodson, another post doctoral fellow, the third task (Months 12-24: If the ER mRNA binding protein is the MCF-7 homologue of the 155 kD *Xenopus* mRNA binding protein, I will clone this protein and investigate its properties.) will require significantly less time than was originally expected. Since the 155 kD *Xenopus* mRNA binding protein was recently identified as vigilin in our lab, the cDNA for this protein has already been cloned. I will still need to investigate it's binding properties if I find evidence that it plays a role in ER mRNA stability.

The forth task (Months 1-24: I will determine whether estrogen and phorbol esters regulate the stability of mRNA through a common mechanism.) will continue to be carried out throughout the remainder of this project.

CONCLUSIONS

The work to date shown in this annual report, represents an essential investment of time and preparation for setting up a new experimental approach which will resolve the ambiguities which have plagued this area of research. We anticipate writing up this methodology for publication in the near future and expect it to be widely used. This experimental design combines the effectiveness of the tightly controlled tetracycline regulated vector expression system with the sensitivity of QT-RTPCR technology modified for use with samples from transfected cells.

The results of my AMD experiments followed by Northern blots for ER mRNA detection were effective and informative for a number of reasons. These studies demonstrated that both estrogen and TPA are capable of down regulating ER mRNA in MCF-7 cells and demonstrated that the MCF-7 cells that I will use for my future experiments will respond in an expected fashion to these agents. This information was essential and will be critical when evaluating the future results of the QT-RTPCR experiments performed on ER mRNA produced from transiently transfected expression vectors in both estrogen treated and untreated MCF-7 and 231N cells. The Northern blot experiments also demonstrated the need to use the most sensitive (QT-RTPCR) mRNA detection system currently available. A 2.0 kb ER mRNA, which was generated after transiently transfecting the TET or CMV-hER expression plasmid into MCF-7 cells, could not be detected through conventional Northern blot assays. Finally, the experiments reiterate the need for developing a system which can directly address the effects of estrogen and TPA on ER mRNA stability without the use of general transcriptional inhibitors which can often complicate the questions raised in stability experiments.

A necessary amount of time was also spent attempting to find the most efficient method of transiently transfecting MCF-7 cells. Despite the sensitivity of RTPCR methods, accurate mRNA quantitation requires larger amounts of starting mRNA (44). Therefore, the more effectively the MCF-7 cells can be transfected (and subsequently used to generate ER mRNA), the more reliably it will be possible to detect differences in mRNA levels between treated and untreated cells.

Finally, while Maria Acena, an MD/PhD student, and I have been working on setting up the QT-RTPCR system in Dr. David Shapiro's lab, I have created all of the necessary full length ER constructions and internal standards. These constructions required a fair amount of effort due to the difficulty in creating a 6.4 kb PCR product using RT-PCR technology as well as the making of a construction which can generate a full length ER mRNA which can be distinguished from the endogenous ER mRNA in MCF-7 cells. Nevertheless, I can now test the effects of estrogen and TPA on ER mRNA stability in MCF-7 and 231N human breast cancer cells using the tetracycline regulated expression vector system followed by QT-RTPCR. The results of these experiments will then allow us to proceed in determining the precise region of the ER mRNA which is significantly influenced by the destabilizing effects of estrogen and TPA and the proteins responsible for interacting with the ER mRNA.

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APPENDICES

(See accompanying figures 1-9 and legends)

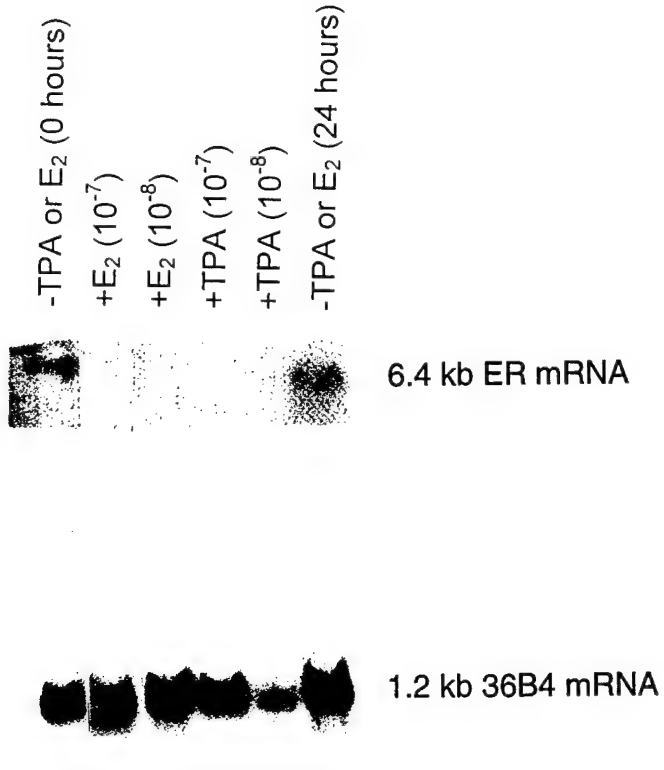


Figure1. ER mRNA levels are significantly lowered in MCF-7 cells in the presence of estrogen (100 and 10nM) or TPA (100 and 10 nM) for 24 hours. Northern blots of mRNA levels for ER and 36B4 are shown above. The autoradiograph shown is a representative of three independent experiments. 36B4 mRNA levels were unaffected by estrogen or TPA and serve as an internal control for differences in gel loading.

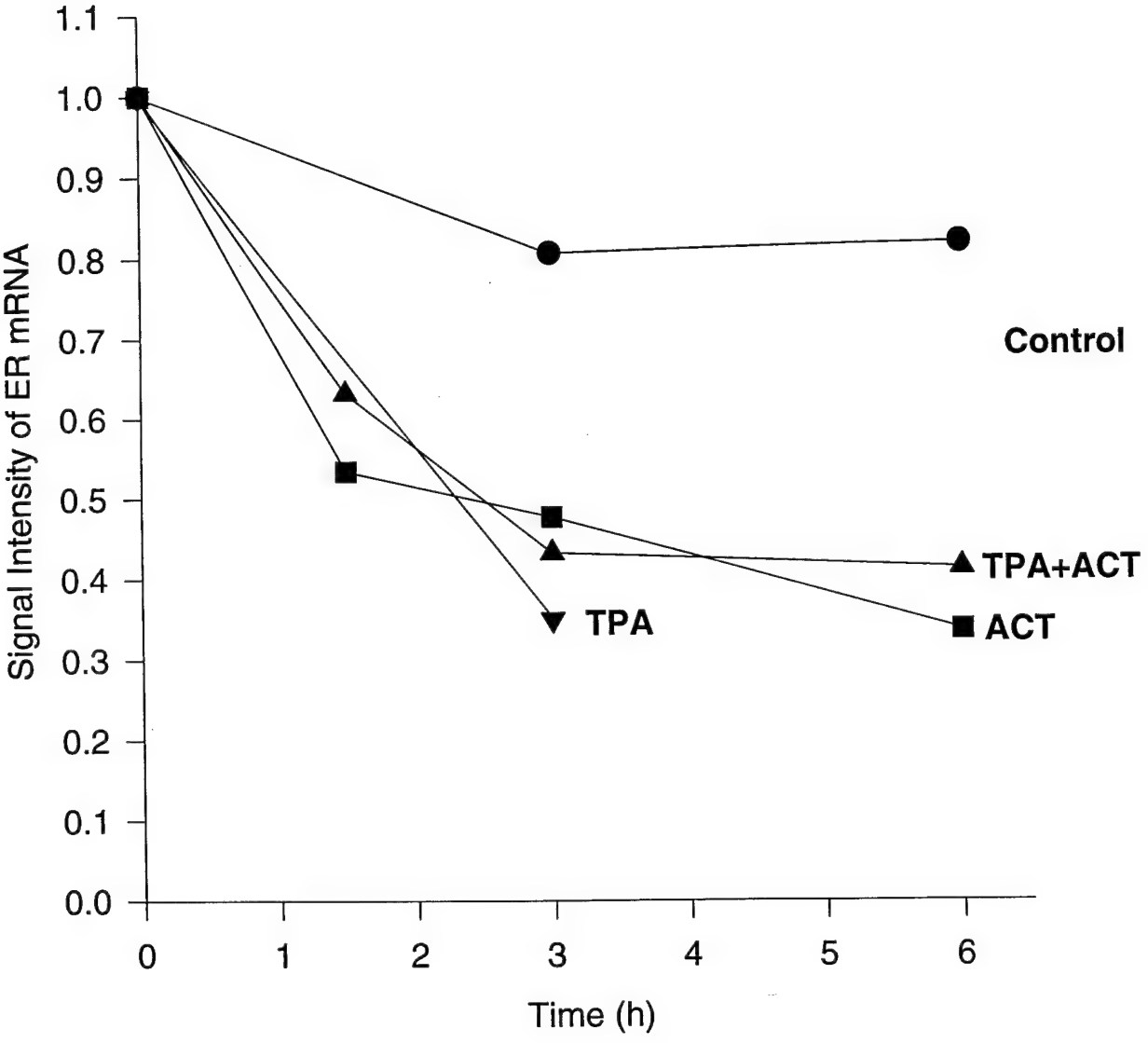


Figure 2. TPA and/or AMD significantly decrease ER mRNA in MCF-7 cells. MCF-7 cells were incubated with TPA (10nM) and/or AMD (4.0 μM) for the time periods indicated above. 5 μgs of total RNA are loaded on to the gel for each sample. Northern blot analysis of ER mRNA was performed and quantitated using a PhosphorImager. 36B4 mRNA served as an internal control.

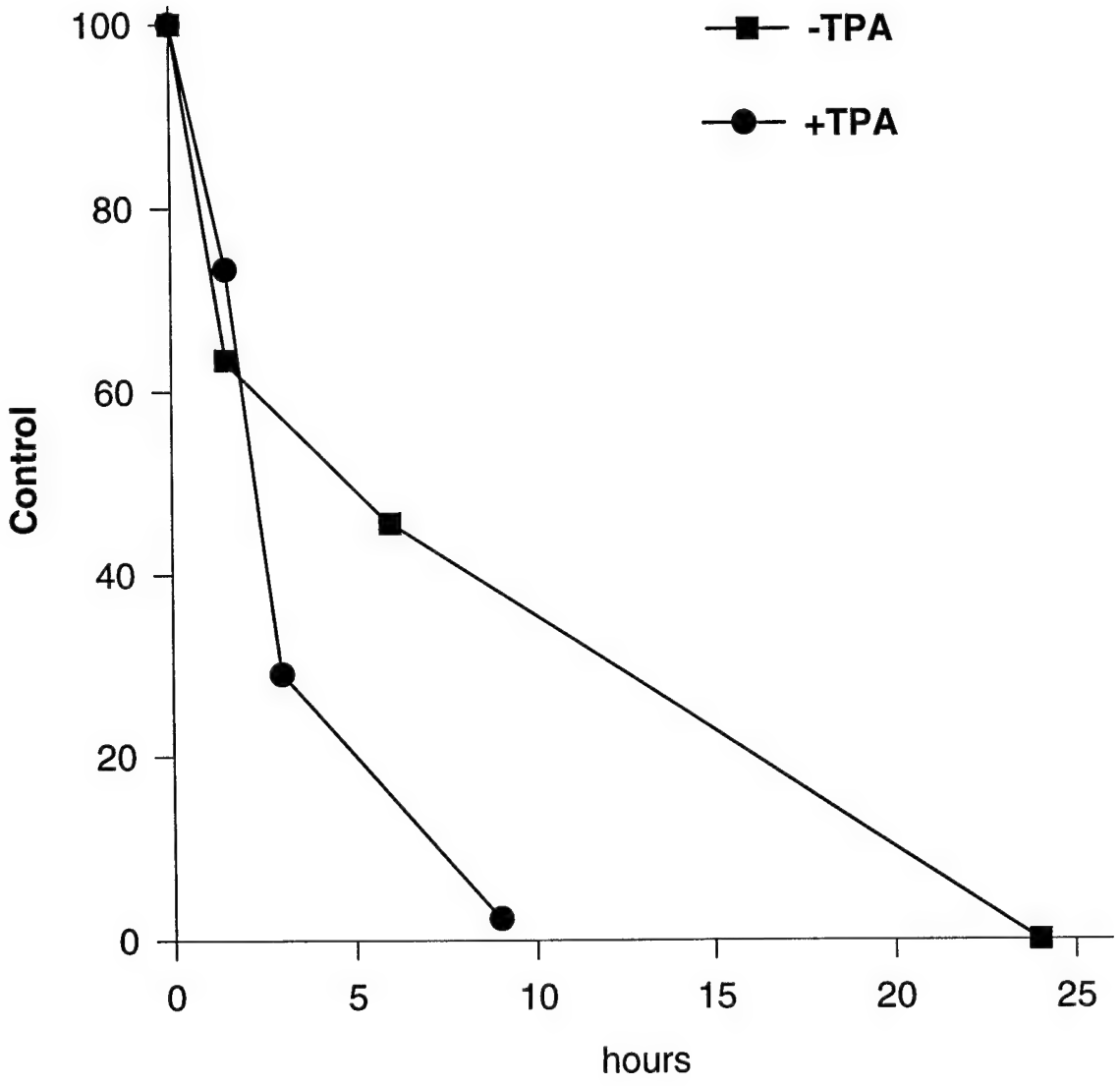


Figure 3. ER mRNA half-life in the presence and absence of TPA. MCF-7 cells were treated for 24 hours with TPA (10 nM) or vehicle, transcription was stopped by the addition of 4 μM AMD. Values are presented as percent of control. Northern blot analysis of ER mRNA was performed and quantitated using a PhosphorImager. 36B4 mRNA served as an internal control.

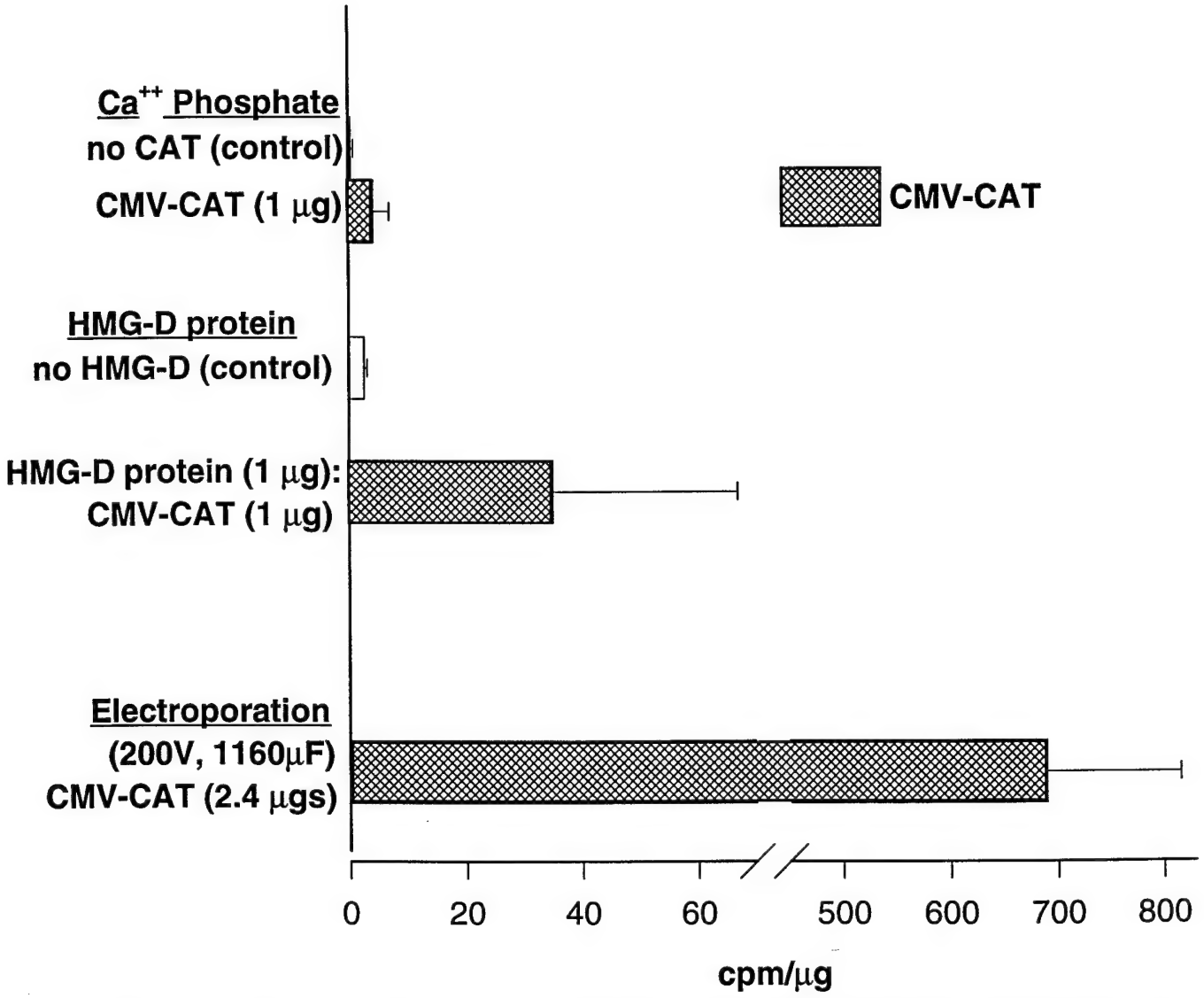


Figure 4. Electroporation is a more efficient method for transiently transfecting MCF-7 cells when compared to the calcium phosphate precipitation method and the HMG-D non-viral gene transfer method. In all transient transfection experiments (Figs. 4-7), unless indicated, 1 μg of CMV-CAT plasmid is transfected, CAT samples are heat treated, and CAT activity is measured as cpm/μg (of protein). After harvesting, MCF-7 cell extracts are then subjected to CAT assays as previously described (42). n=3 for all experiments.

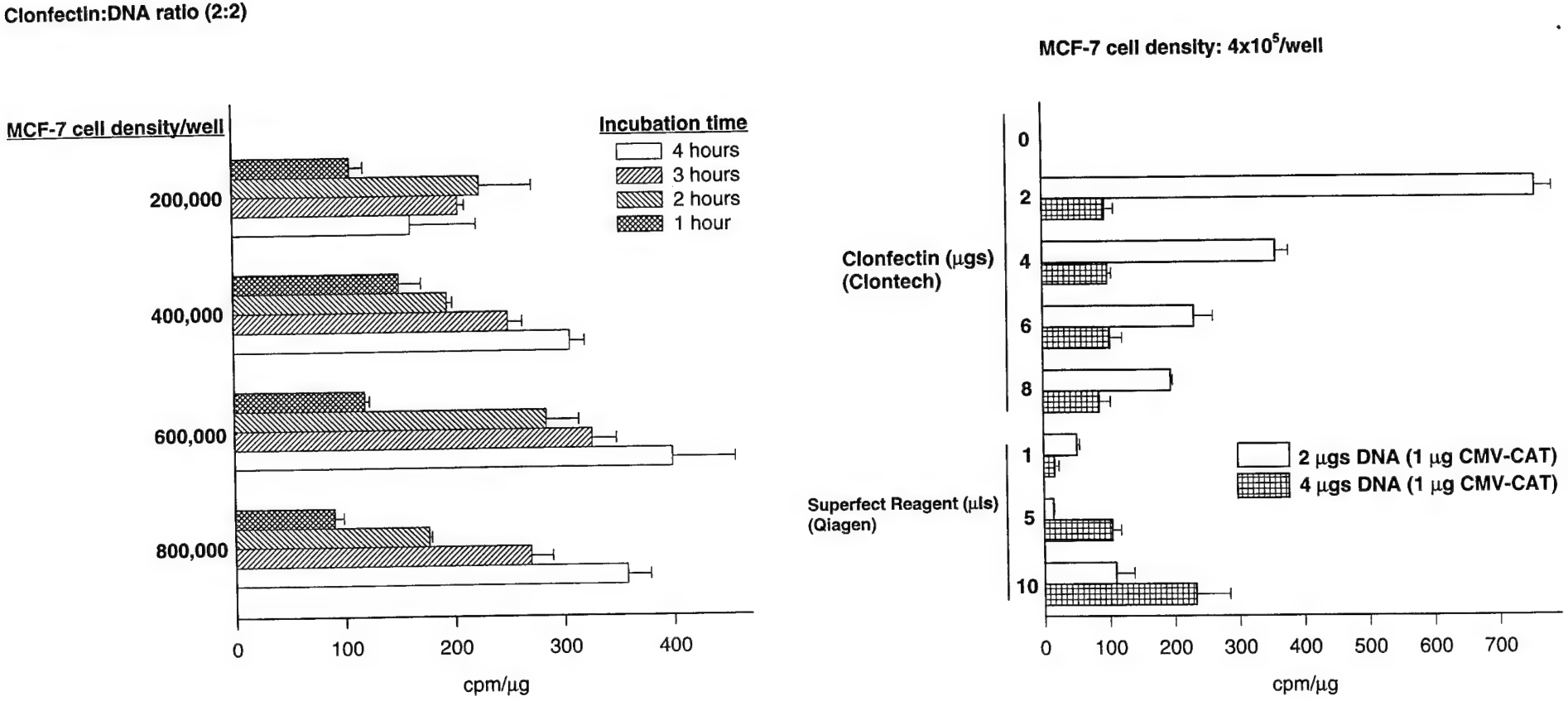


Figure 5. Left panel: Time-dependent increases in CAT activity when MCF-7 cells are incubated with 1:1 ratios of Clonfectin to CMV-CAT cDNA. MCF-7 cell density prior to transfection, does not significantly effect transfection efficiency except at the lowest density of 200,000 cells/well. Right panel: Clonfectin is a more efficient transient transfection reagent when compared to Superfect reagent in MCF-7 cells as determined by significant differences in CAT activity. Reagent:DNA ratios were optimized for both Clonfectin and Superfect. After harvesting, MCF-7 cell extracts are then subjected to CAT assays as previously described (42). n=3 for all experiments.

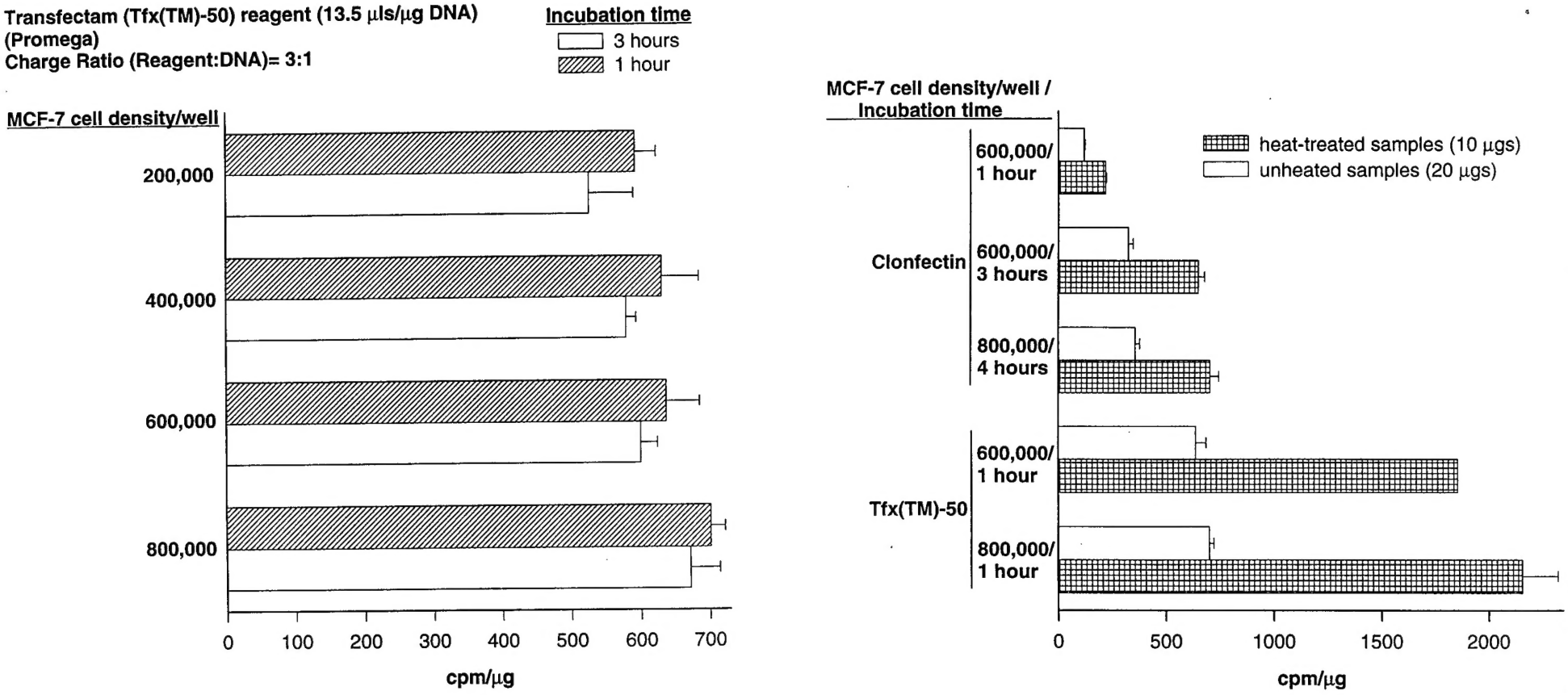
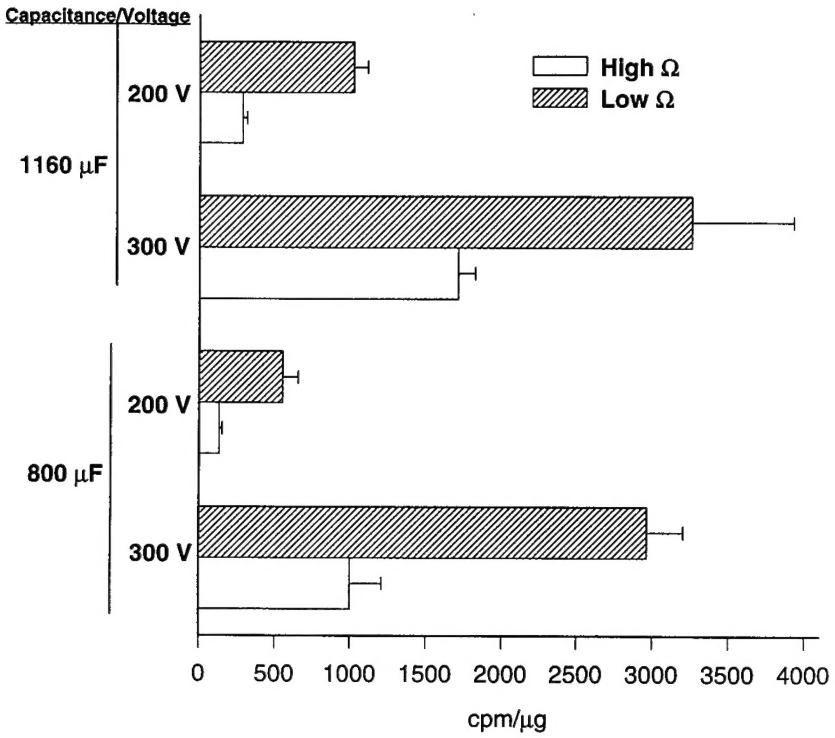


Figure 6. Left panel: Transfectam reagent transiently transfects MCF-7 cells with the same efficiency regardless of incubation time and cell density. Right panel: Transfectam is the most efficient of the "liposomal" reagents tested for transiently transfecting MCF-7 cells. Moreover, heat-treating CAT samples prior to assay (37°C for 2 hours) significantly increases CAT activity. After harvesting, MCF-7 cell extracts are then subjected to CAT assays as previously described (42). n=3 for all experiments.



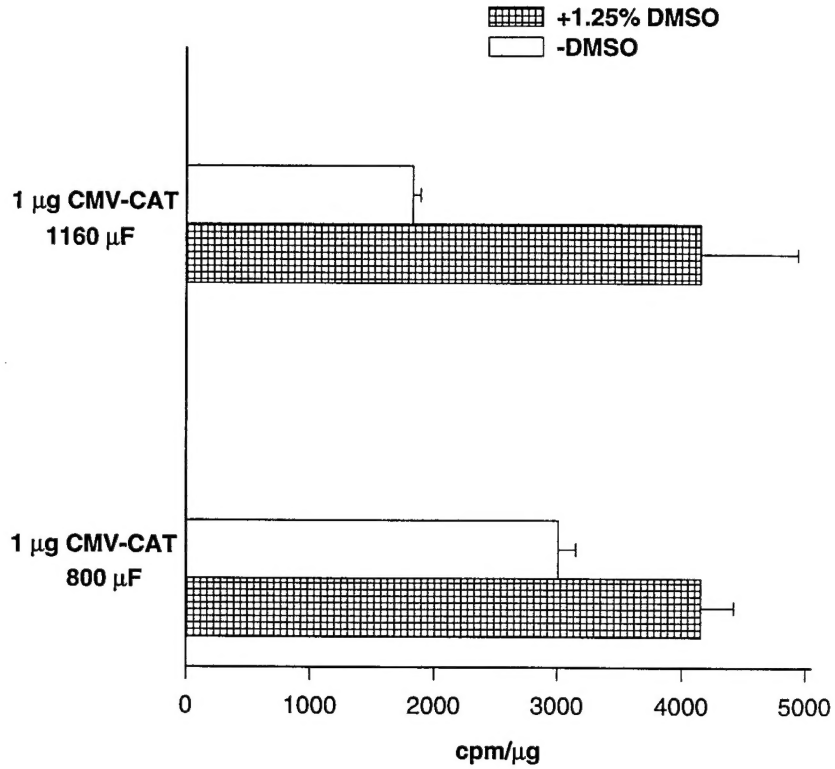


Figure 7. Left panel: A low resistance setting on the electroporator increases efficiency for MCF-7 cell transient transfections when using the electroporation method. Right panel: DMSO enhances transient transfection efficiency in MCF-7 cells when using the electroporation method. After harvesting, MCF-7 cell extracts are then subjected to CAT assays as previously described (42). n=3 for all experiments.

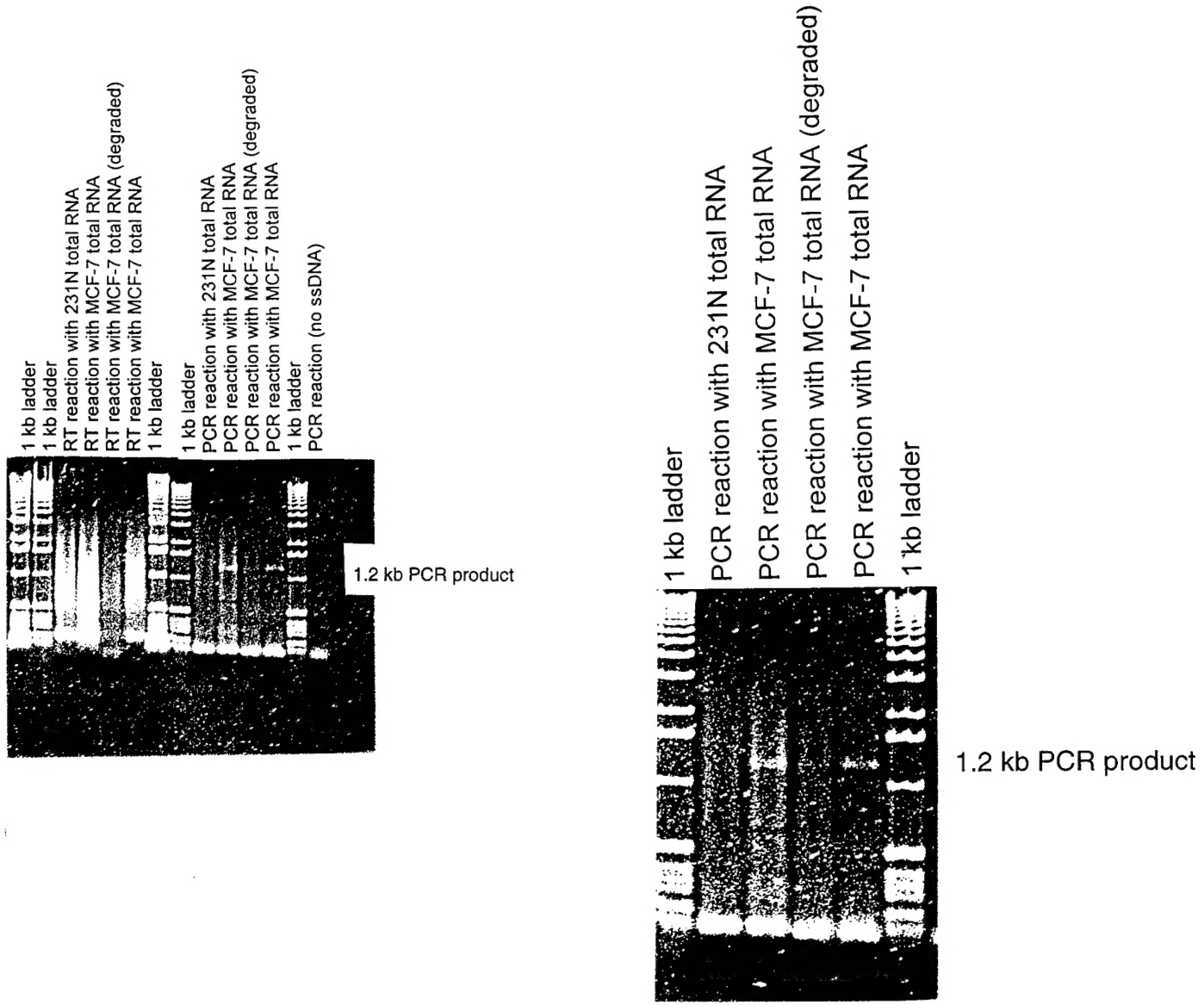


Figure 8. 231N cells do not contain detectable levels of ER mRNA with RT-PCR. Untreated 231N and MCF-7 cells had their total RNA extracted and reversed transcribed using a 3' primer specific for ER mRNA. The subsequent PCR reaction was performed using the same 3' primer and a 5' primer also specific to ER mRNA. A 1.2 kb PCR product was generated from total RNA from MCF-7 cells but not from 231N cells. The RT reaction created no distinct bands from any of the samples. Integrity of RNA samples was determined by visualizing the ribosomal bands from each sample and measuring the 260/280 ratio for each sample. All RNA samples had 260/280 ratios >1.8 except (degraded) which was 1.23.

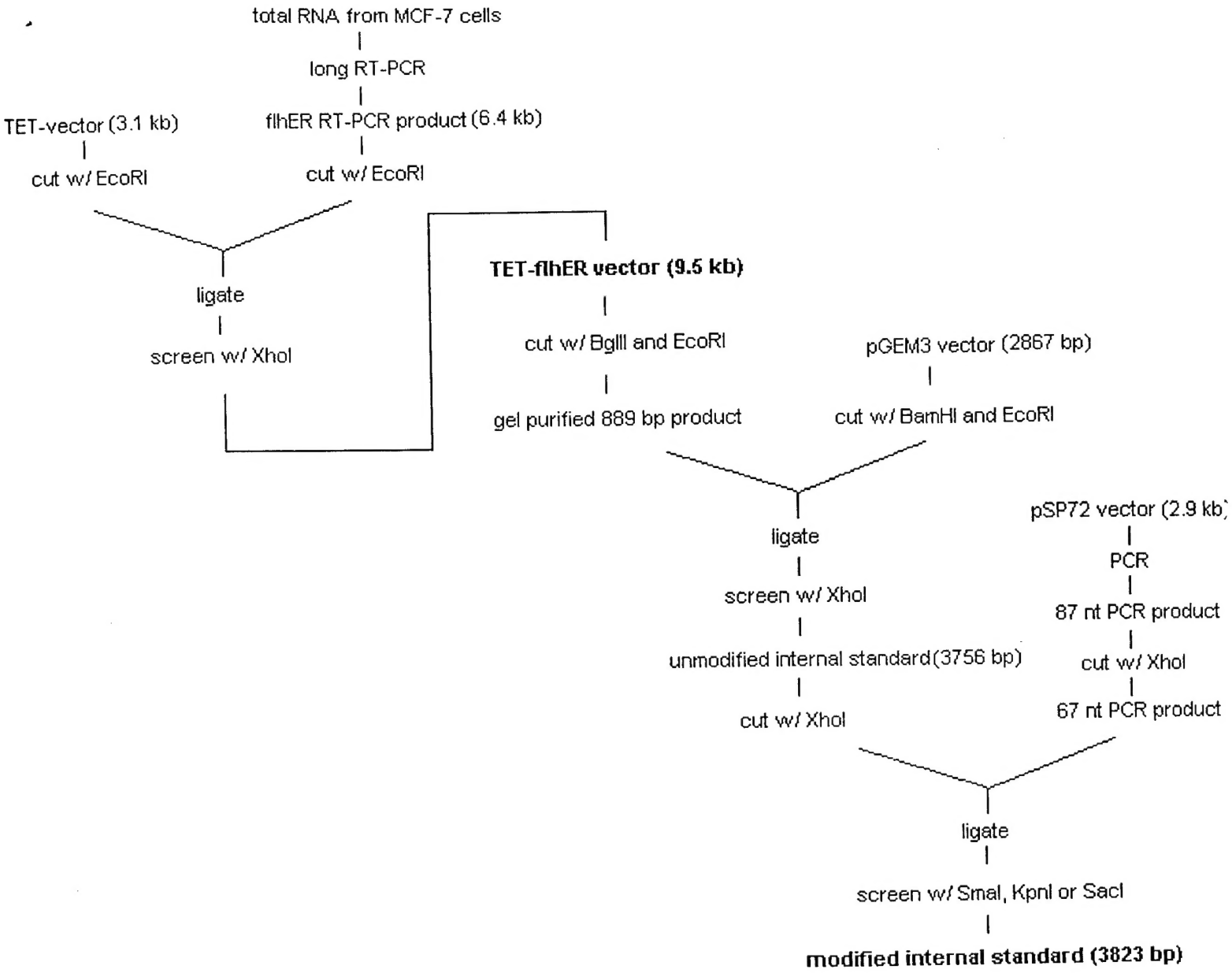


Figure 9. Schematic drawing showing the construction strategy for cloning the initial TET-flhER plasmid and it's internal standard. Screening for the TETflhER construction also included digests with BamHI, BglII, BstEII, EcoRI, and KpnI. Details of the constructions are further elaborated in the Results and Discussion section.